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13. ABSTRACT *(Maximum 200 words)*

1. To define their carbohydrate binding specificity, a variety of *Vibrio cholera* strains were tested for their ability to (a) hemagglutinate red blood cells in a carbohydrate-inhibitable manner; (b) bind radiolabeled multivalent carbohydrate ligands; and (c) aggregate in response to multivalent carbohydrates. Although hemagglutination and aggregation were demonstrated, reproducible carbohydrate specificity was not apparent.

2. Binding of *E. histolytica* membranes to specific carbohydrates was characterized. High affinity binding to clustered, multivalent GalNAc residues was demonstrated. Highest affinity was obtained when ≥20 GalNAc residues were synthetically clustered by covalently attaching them to a carrier protein (bovine serum albumin). Binding of GalNAc to the *E. histolytica* lectin required the 3- and 4-position hydroxyls, as demonstrated using synthetic deoxy analogs of GalNAc.

3. βGlcNAc-specific hexosaminidase was characterized in *E. histolytica* membranes.

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Contents

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Lists of Figures and Tables

CHAPTER 1: *Vibrio cholera* Binding To Carbohydrates

Statement Of Problem

Vibrio cholera infects humans as a food or water contaminant, causing severe dehydration and ataxia. Fluid imbalance is caused by the production of an enterotoxin consisting of two subunits, A and B. Both units are required for the activation of adenylate cyclase, and thus fluid deregulation. Subunit B serves as the anchor to the cell surface and subunit A is responsible for the active release of fluid from mammalian cells.(1)

In addition to a toxin, Vibrio cholera carries fucose, mannose, and n-acetylglucosamine inhibitable hemagglutinins which may serve as colonizing molecules within the bowel.(2,3)

Studies of hemagglutination involving environmental strains of V. cholera have shown that the presence of these adhesins depends on the nutritional and stress status of the organisms' immediate environment. The production of some adhesins has been shown to be dependent on protein synthesis free of cell division.(4) It is questionable whether or not the adhesins are necessary for disease production in humans in all cases, or whether they are regulated simply in response environmental conditions in the bowel as part of a survival mechanism, reminiscent of environmental strains.

Cholera toxin (CT) is produced by strains of Vibrio cholera that belong to serogroup 01 or the recently reported serogroup, 0139. Both carry the potential to cause epidemic cholera. V. cholera from the 01 serogroup may be from any of the following serotypes, Classical, El Tor, Ogawa, or Inaba. Classical biotype strains have two copies of the toxin operon (ctx) at separate loci on the bacterial chromosome. Most El Tor strains have a single copy operon. Some El Tor strains have multiple copies of the ctx operon on tandemly repeated genetic elements that are capable of transposition and site-specific recombination. These tandemly repeated elements vary in size due to differences within the elements in copy number of a smaller repeated sequence called RS1.(1)

Mutants producing hypotoxigenic mutants (1000-fold lower levels of toxin than wild type) mapped to a locus called toxR. Hypertoxigenic strains have also been isolated and characterized. Expression of the toxin operon is environmentally regulated by the transcriptional activator toxR as part of a regulatory cascade that controls the expression of many virulence genes in V cholera. A tandemly repeated sequence is located upstream of the ctx operon and serves a s a recognition sequence for the toxR gene product. Most environmental factors have significant effects on control of CT production.(1)

Work involving the Tcp colonization pilus has shown that toxR also regulates, via a transcriptional activator, the coordinate production of cholera toxin and the tcpA encoded pilus.(5,6,7) This was shown to occur in an 0395 strain grown on laboratory media and in on another strain using in vivo experimentation.(7) One strain used in our experiments, JJM43, carries a deletion mutation of the toxR structural gene (25 bp deletion) and subsequently a colonization defect in animals.(8) However, a small amount of tcp-A encoded pilus was still made by the strain in the absence of a functional toxR gene, suggesting multigene regulation.

It has been suggested that ToxR may be a global regulatory protein capable of producing coordinate and opposite shifts in the expression of two distinct families of genes. One family may contain genes involved in virulence, the other family might include genes important to the survival of V cholera in marine animals or water ecosystems.(7) N-acetyl-d-glucosamine specific cell

associated hemagglutinin (47 kDa) has been reported to occur on both V. cholera 01 and non-01 strains, suggesting its significance as an environmental survival mechanism. This adhesin thus becomes significant from an epidemiological point of view.(3)

Genetic control of the production of both tcp and toxin subunit B seems to be more dependent on the presence of the promoter than the location of the genes themselves. This has been shown by J. Mekalanos in the mutant constructs made from 0395 N1 - organisms producing both B subunit and TCP. 0395-N1 ctxB on its own chromosome; 0395-N1 with plasmid pJM290.2 carrying several copies of the ctx B gene under the control of its own promoter; 0395-N1 containing the plasmid pJM 23 Tac.E carrying the ctxB gene under the control of the constitutive Tac promoter, all produced TCP in parallel with toxin B. The strain of 0395-N1 used in our experiments carries ctx B on its own chromosome controlled by a chromosomally regulated promoter. Further intricacies in the regulation of adhesive properties occur with "acf" genes.

In Vibrio cholera, acfA,C, and D genes are involved in the synthesis of a colonization factor and their expression is under the control of toxR, the cholera toxin transcriptional activator. These acf genes are clustered on a 5 kb region and the acfA and acfD genes are transcribed divergently. The translation sites are separated by 173 bp. When carried by the chromosome, the acfA-acfD intergenic region was found to contain cis-acting elements necessary for the environmental regulation of both the acfA and acfD promoter.(9)

This project asked whether or not it is possible to capture V. cholera strains by virtue of their ability to adhere to structures on the surface of mammalian cells. In order to assess this ability, a series of neoglycoproteins was synthesized (10) and tested for their ability to cluster (by virtue of adhesin) both wild type V. cholera 01 and mutants deficient in the ability to synthesize the A subunit of cholera toxin, prepared by deletion of portions of the genome.

Materials And Methods

V. cholera strain 0139 MES-1, was obtained from Dr. William Petri, Jr., University of Virginia. Approximately 1 x 10^8 per ml organisms were recovered from a formalin treated preparation. These organisms were tested for their ability to hemagglutinate fixed red blood cell types, chicken, rabbit, sheep, and human A,B,O. Fresh human A,B, and O were also used. Agglutination was observed using fixed rabbit and fixed human O cells at a concentration of 1 x 10^7/ml. Approximately 5 x 10^7/ml organisms were used. Serial two fold dilutions of organisms, in V-bottom microtiter plates, began at 1 x 10^7/ml and went down to 5 x 10^3/ml. Plates were incubated overnight at 4 C. Hemagglutination titers for both rabbit and human O cells was 5 x 10^4 organisms/ml. PBS with 5 mg/ml BSA was used throughout.(11,12)

Live mutant strains were obtained from DR. John Mekalanos, Harvard University. All were deficient in their ability to produce cholera toxin subunit A and all were from serogroup 01. Classical serotypes included, 0395-N1, TCP2(tcpA-), JJM43(toxR-), and 0395-N1 mot-51 (nonmotile). Strains from the 0139 serotype included Bengal 3 and Bengal 15 mot-. Strains from the El Tor serotype included Bah-3, Bah-15 mot-, Peru-3, Peru-14, and Peru 15 mot-. Unknown serotypes included Bang-3 and Bang-15. Wild type strains included 0395 (parent to 0395-N1 and JJM43), E7946 (parent to Bah-3 and Bah-15), C6709 (parent to Peru-3, Peru-14, and Peru-15), and P27459 (parent to Bang-3 and Bang-15).

The 15 mutant strains were grown on LB plates and stored at -70 C in nutrient broth containing 5-20% glycerol. Prior to the hemagglutination assay, organisms were grown on LB plates and transferred to 20 mls LB broth at pH 6.5-7.0. Broth cultures were grown for 4 hours or overnight at room temperature with aeration.

Results

Hemagglutination Assays-- All 13 mutant strains agglutinated fresh human O cells at titers ranging from 2.5×10^6 to 1.9×10^4 organisms/ml when using red cells at 1×10^7/ml. No Ca^{+2} or Mg^{+2} dependencies were observed for agglutination to occur. Although agglutination was consistent day to day, break points varied per organism per day. With this in mind, sugar inhibition studies were undertaken and a non-sugar control was run with every inhibition test every day.

Before inoculating organisms into red cell wells, several concentrations of each sugar were incubated with the washed organisms in PBS/10% BSA for 45 min. at 4 C. L-Fucose and D-mannose were used at concentrations of 0.5 mM, 10 mM, 20 mM, 40 mM, 60 mM, and 80 mM. No inhibition effects were seen when using concentrations ranging from 0.5 mM to 20 mM, but inhibition of 1-2 dilutions was observed in the 40-80 mM range.

Although a pattern of inhibition could be observed with each assay, this pattern varied day to day. Results using fresh human O cells are shown in *Table I.*

The same assay was repeated using fresh human O red cells. It was found that in this assay, more organisms were needed to get visible reads. Results of the assay are shown in *Table II.*

We were unable to reproduce hemagglutination results day to day and were unable to reproduce Dr. Mekalanos' inhibition data using fixed chicken cells or fresh human O cells. Nonspecific inhibition was observed using sugars not related to mannose of fucose. Therefore, radiolabeling of neoglycoproteins for adherence assays was undertaken.

Radioligand Binding Assays-- The following neoglycoproteins were labeled and resulted in the respective specific activities; $GlcNAc_{10}BSA$, 250.5 uCi/nmol, $Fuc_{28}BSA$, 169.4 uCi/nmol, and $Man_{28}BSA$, 660 uCi/nmol. $GalNac_{39}BSA$ was used as a control for nonspecific binding. Dilutions of each labeled neoglycoprotein were made and diluted to a concentration giving approximately 100,000 counts per 10 µl on the gamma counter. Organisms were used initially at various concentrations to determine an optimal binding concentration.

Organisms Bah 3 and Bengal 3 were grown in LB broth with 5 grams salt per liter for 4 hours rotating at room temperature. Centrifuge and resuspend pellet in BSH, pH 7.4, osmolarity 340, containing 2 gm per liter lactose, no glucose, and 1 mg/ml BSA. Organism suspensions were made at 10^9, 10^8, 10^7 and 10^6 CFU/ml and held on ice while preparing the binding assay tubes. Binding assays were rotated at 4 C for 1 hr in 12 x 75 mm glass tubes. Glass fiber filters were pre-soaked in 10 mM Hepes, pH 7.4, containing 1 mg/ml BSA. Tubes were placed on the Brandel harvester, diluted rapidly with cold Hepes buffer, 10 mM, pH 7.4 and drawn through the filters. Filters were placed in tubes and quantified using the gamma counter. One minute counts were obtained. Controls consisted of 10 µl of labeled neoglycoprotein for input counts x 2, and filter controls having 10 µl input in buffer, pulled through the filter x 3 for each condition.

Results show no apparent difference in binding dependent on the concentration of organisms used (*Fig. 1).* Inhibition binding studies were also performed as described above with the addition of inhibiting (unlabeled) neoglycoproteins added 10 min before labeled neoglycoproteins. Unlabeled Man_{28}BSA at was used at 26.5 nM and unlabeled Fuc_{28}BSA at 191.6 nM. For Bah 3 mannose binding, 3.5% of the total input remained on the filter and 1.4 % of total signal remained after blocking. For Bengal 3 and fucose binding, 4.4% of the total input remained on the filters and 1.8% remained after blocking (*Fig. 2).* Binding and inhibition studies were continued for reproducibility and signals remained minimal but consistent. Specificity of inhibition is shown in *Fig. 3.*

Optimization of Signal for Binding Assays-- Attempts were made to optimize the signal to a level needed for affinity determinations. The following adjustments were tried. Polycarbonate 12 x 75 tubes were used to avoid possible sticking to glass of either the neoglycoproteins or the organisms. Organisms were grown at room temperature, rotating, for four hours and overnight. Concentrations of Ca^{+2} and Mg^{+2} in the assay buffer ranging from 50 to 150 μM were tried. Organisms grown in LB containing 2.0 % lactose, 0.2% lactose, 2% casamino acids, and LB containing 10 gm/L NaCl instead of 5 gm/L NaCl. Buffer substitutes included KRT and 8.5% NaCl. Additionally all other mutant strains were tested for binding. In all cases very little radioligand binding was observed, ranging from 0.1 to 1.0% of total input.

Agglutination Assays with Gram Stain-- Since optimization of the assay conditions for radioligand binding failed, the ability of the neoglycoproteins to induce microbe agglutination was determined using wild type *V. cholera* strains E7946, and E27459. Organisms were grown overnight in 20 ml LB containing 0.2% sucrose, collected by centrifugation and resuspended in 5 ml Hepes 0.01 M, pH 7.3, 1 mg/ml BSA. From the 5 ml resuspension, 100 μl was added to 500 μl buffer, and 10 μl of neoglycoprotein was added. The final concentration of organisms was 1.6 x 10^9CFU/ml. Concentrations of Man_{28}BSA 0.28 nM, Fuc_{28}BSA 3.2 nM and $GlcNAc_{10}$BSA, 4.2 nM were used to induce microbe agglutination.

Sampling entailed making a gram stain of a dedicated assay tube for each condition at hourly intervals of (1-5 hr.) or overnight. Results showed that each neoglycoprotein aggregated each organism a unique time period during the assay. Typically, the 3 to 5 hour time period showed the most aggregation. Diminished aggregation was seen at 5 hours and overnight. Day to day variation occurred in the kinetics and extent of clumping.

As a follow up for this data, attempts were made to develop a spectrophotometric assay sensitive enough to see clumping. Assays were performed as before and read at 600 nm. No difference between control and test was seen at 3, 4 or 5 hour time periods and gram stains showed considerable clumping. This approach was abandoned due to lack of sensitivity.

Gram stain results were complied for all wild type and mutant strains at 3,4 and 5 hr using Man_{28}BSA, Fuc_{28}BSA, $GlcNAc_{10}$BSA, and $GalNAc_{39}$BSA. Neoglycoproteins were used at 10 nM and blocking sugars at 50 mM. Distinct patterns of clumping were observed which, however, were not reproducible (*Table III*). Assays which included soluble monosaccharide inhibitors are shown in *Table IV.*

Filter Assay for Aggregation-- Attempts were made to use 10 micron pore filters with uniform holes ("isopore" bisphenol polycarbonate) to collect and quantitate aggregates using radiolabeled bacteria. However, there was minimal radiolabel retained on the filter, even when parallel gram stains showed considerable aggregation. Results led us to conclude that disaggregation occurred as the cells came in contact with the filters.

Conclusion

Despite extensive efforts and multiple assay systems, we were unable to reproducibly and convincingly demonstrate carbohydrate-specific adhesion or aggregation of *V. cholera* strains. Since there have been previous reports of carbohydrate hemagglutination, we conclude that either the background binding was too high to allow specific binding to be revealed, or that the probes (subsequent to hemagglutination) were not complex enough (in carbohydrate structure) to elicit a binding response of sufficient affinity to detect.

Literature Cited

1. Hirst, T (1995) "Biogenesis of Cholera Toxin and Related Oligomeric Enterotoxins." In *Bacterial Toxins and Virulence Factors in Disease*. J. Moss, B. Iglewski, M. Vaughan, and A. Tu, eds, Marcel Dekker, Inc. New York, N.Y. pages 123-184.

2. Jonson, G., Sanchez, J., and Svennerholm, A. (1989) "Expression and Detection of Biotype-associated Cell-bound Haemagglutinins of Vibrio cholerae 01," *Journal of Clinical Microbiology* **135**, 111-120.

3. Sasmal, D., Guhathakurta, B., Ghosh, A.N., Pal, C.R., Datta, A. (1992) "N-Acetyl-D-glucosamine-specific lectin purified from Vibrio cholerae 01," *FEMS Microbiology Letters*, **98**, 217-224.

4. Yu, C., Lee, A.M., and Roseman, S. (1987) "The Sugar-specific Adhesion/Deadhesion Apparatus of the Marine Bacterium Vibrio furnissii is a Sensorium that Continuously Monitors Nutrient Levels in the Environment," *Biochem. Biophys. Res. Commun.* **149**, 86-92.

5. Shaw, C.E., Peterson, K.M., Sun, D., Mekalanos, J.J., Taylor, R.K. (1989) "TCP Pilus Expression and Biogenesis by Classical and El Tor Biotypes of Vibrio cholerae 01," In *Molecular Mechanisms Of Microbial Adhesion: Proceedings Of The Second Gulf Shores Symposium*. Springer-Verlag, New York, N.Y. pp. 23-35.

6. Shiloach, J., Walle, M., Taxman, E., and Mekalanos, J.J. (1992) "Correlation between Cell Agglutination, TCPA and Cholera Toxin B Subunit Production by High Level Producing Strains of Vibrio cholera," In: *Bacterial Protein Toxins*. Jena, New York, pp. 194-196.

7. Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987) "Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin," *Proc. Natl. Acad. Sci. USA* **84**, 2833-2837.

8. Jacob, A., Sinha, V.B., Sahib, M.K., Srivastava, R., Kaper, J.B., and Srivastava, B.S. (1993) "Identification of a 33 kDa antigen associated with an adhesive and colonizing strain of Vibrio cholerae El Tor and its role in protection," *Vaccine* **11**, 376-382.

9. Parsot, C., and Mekalanos, J.J. (1992) "Structural Analysis of the acfA and acfD Genes of Vibrio cholerae: Effects of DNA Topology and Transcriptional Activators on Expression," *J. Bact.***174**, 5211-5218.

10. Lee, Y.C., Stowell, C.P. and Krantz, M.J. (1976) "2-Imino-2-methoxyethyl 1-thioglycosides: New reagents for attaching sugars to proteins," *Biochemistry* **15**, 3956-3963.

11. Roy, K.D., Dasgupta, C., and Ghose, A.C. (1989) "Hemagglutination and Intestinal Adherence Properties of Clinical and Environmental Isolates of Non-01 Vibrio cholerae," *Applied and Environmental Microbiology* **55**, 2403-2406.

12. Hanne, L.F., and Finkelstein, R.A. "Characterization and Distribution of the Hemagglutinins Produced by Vibrio cholerae," *Infection and Immunity* **36**, 209-214.

Table I

	TCP2	JJM43	Bang 3	Bengal 3	Bah 3	Peru 3
D-GlcNAc	neg	neg	neg	1+	neg	neg
D-Gal	neg	neg	neg	1+	neg	neg
L-Fuc	1+(4+)	neg(4+)	1+(+-)	1+(+-)	neg(+-)	neg(+-)
D-GlcN	1+	neg	1+	neg	neg	1+
D-Fru	1+	neg	neg	neg	neg	neg
D-Man	1+(+-)	neg(+-)	neg(4+)	neg(4+)	neg(4+)	neg(4+)

( ) inhibition results obtained with fresh chicken erythrocytes using L-Fuc and D-Man (John Mekalanos). 1+ = One two-fold dilution inhibited. 4+ = Four two-fold dilution inhibited. Sugars were used at 80 mM. Organisms grown overnight in LB, centrifuged, and diluted to 2 x 10^8/ml in PBS/BSA for use in the assay. Fixed chicken red cells at 0.5% hematocrit in PBS/BSA(10 mg/ml)

Table II

	TCP2	JJM43	Bang 3	Bengal 3	Bah 3	Peru 3
D-GlcNAc	neg	neg	neg	neg	neg	neg
D-Gal	1+	neg	neg	neg	neg	neg
L-Fuc	3+(4+)	neg(4+)	neg(+-)	neg(+-)	neg(+-)	neg(+-)
D-GlcN	3+	neg	neg	neg	neg	neg
D-Fru	4+	neg	1+	neg	neg	neg
D-Man	4+(+-)	neg(+-)	4+(4+)	neg(4+)	neg(4+)	neg(4+)

( ) Previous results with fresh chicken erythrocytes using D-Man and L-Fuc. 1+ = one two-fold dilution inhibited; 4+ = Four two-fold dilutions inhibited. All sugars at 80 mM. Organism suspensions begin at 2 x 10^9 CFU/ml. Fresh human O red blood cells at 2.5 x 10^7/ml.

Table III

Strain	Date	Incubation Time (hr)	$Man_{35}BSA$	$Fuc_{30}BSA$	$GlcNAc_{10}$ BSA	$GalNAc_{39}$ BSA	Control
E7946	8/10/95	3	-	+	-		-
		4	+++	++	++++		-
	9/6/95	4	-	+/-	+	+/-	+/-
		5	++	+/-	+/-		-
Bah 3	9/13/95	3	++	+++	+	-	-
		4	+/-	+/-	+	-	-
Bah 15	9/13/95	3	+++	++	+++	++	++½
		4	+/-	+++	+	+/-	-
E27459	8/10/95	3	+/-	++	++++		-
		4	+++	+++	+++		-
	9/6/95	4	-	+/-	-	-	-
		5	++	-	++		-
Bang 3	9/13/95	3	+/-	++	+/-	+/-	-
		4				+	+
Bang 15	9/13/95	3	-	-	-	-	-
		4	-	-	-	-	-
	9/6/95	4	++	+++	+/-	-	-
0395-N1	9/13/95	4	++++	++	+	++	-
		5	+/-	-	+++	+	++++
0395-N1-5	9/13/95	4	-	-	-	-	-
		5	-	-	-	-	-
C6709	9/6/95	4	++	+++	+++++	++	++
TCP2	9/13/95	4	++	+	-	+	-
		5	-	-	-	-	-
Bengal 3	9/13/95	4	+++	-	-	-	-
		5	-	++	++	+½	-
Bengal 15	9/13/95	4	-	-	-	-	-
		5	-	-	-	-	-
JJM43	9/21/95	3	++++	+++	++++	+++++	++
		4	+++	++++	++++	++	+++
Peru 3	9/21/95	3	+++++	+++++	+++++	+++++	+++
		4	++++	++++	++++	++++	++++
Peru 14	9/21/95	3	++++	++++	++++	+++	++++
		4	++++	++++	++++	++	++++
Peru 15	9/21/95	3	-	-	-	-	-
		4	-	-	-	-	-

Table IV

Strain	Time (hr)	Control	ManBSA	ManBSA + Man	ManBSA + GalNAc	FucBSA	FucBSA + Fuc	FucBSA + GalNAc	GlcNAcBSA	GlcNAcBSA + GlcNAc	GlcNAcBSA + GalNAc
C6709	3	+++	++	+	++++	+++	++++	+++++	++	+++	++++
	4	++	++++	+	+½	++++	+++	++++	++++	+++	+++++
P27459	3	-	+	+	+/-	+½	-	++	+/-	++	-
	4	-	-	-	++	+	+	++	+	+++	++
E7946	3	++++	+/-	+/-	-	+/-	+	+/-	+	+	-
	4	++++	-	-	-	-	++	+	+	++	-
O395	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	+++	-	-	-

Figure 1: Radioligand Binding of Sugar-BSA conjugates

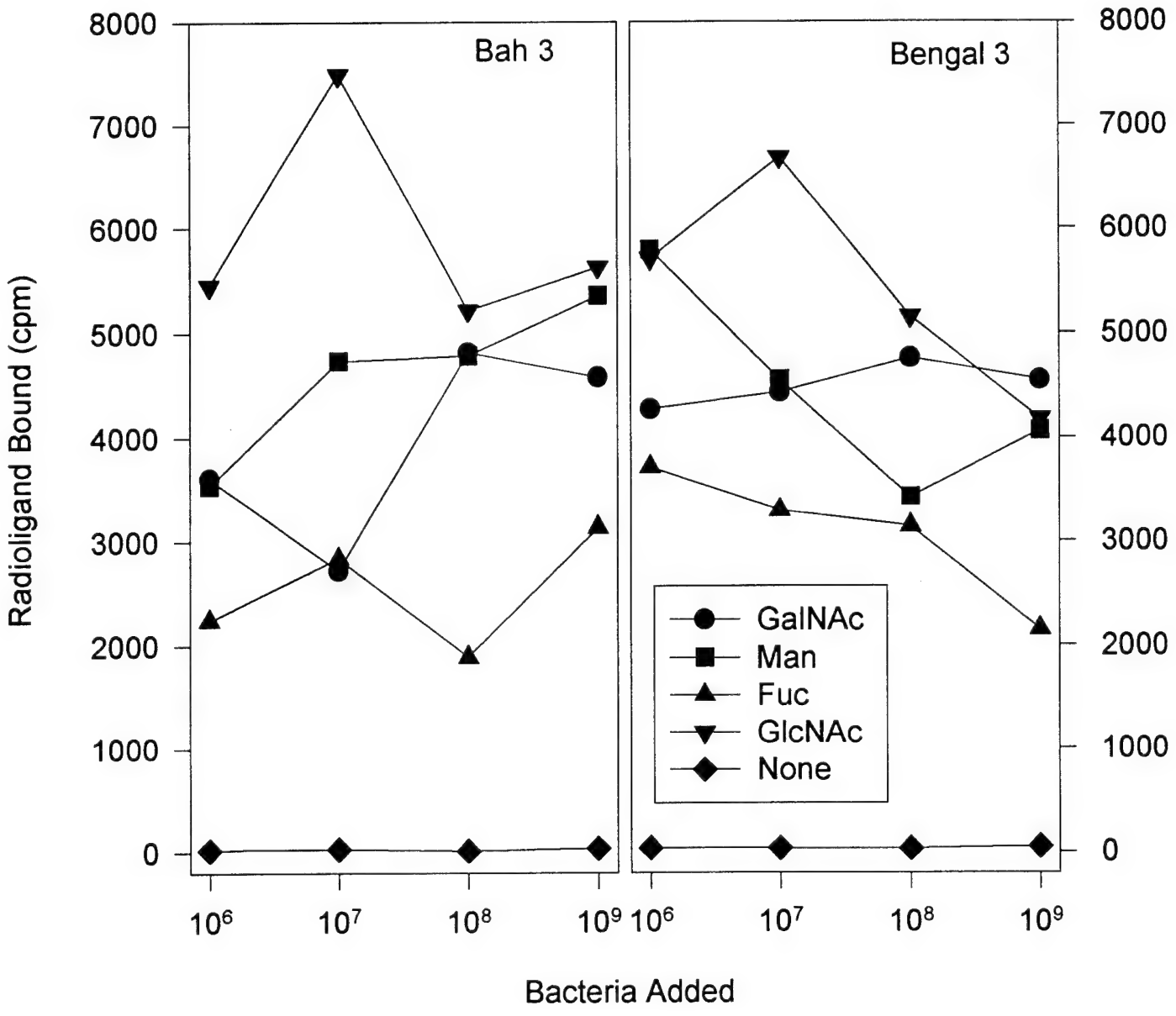


Figure 2: Radioligand Binding of Sugar-BSA conjugates
Effect of excess unlabeled ligand

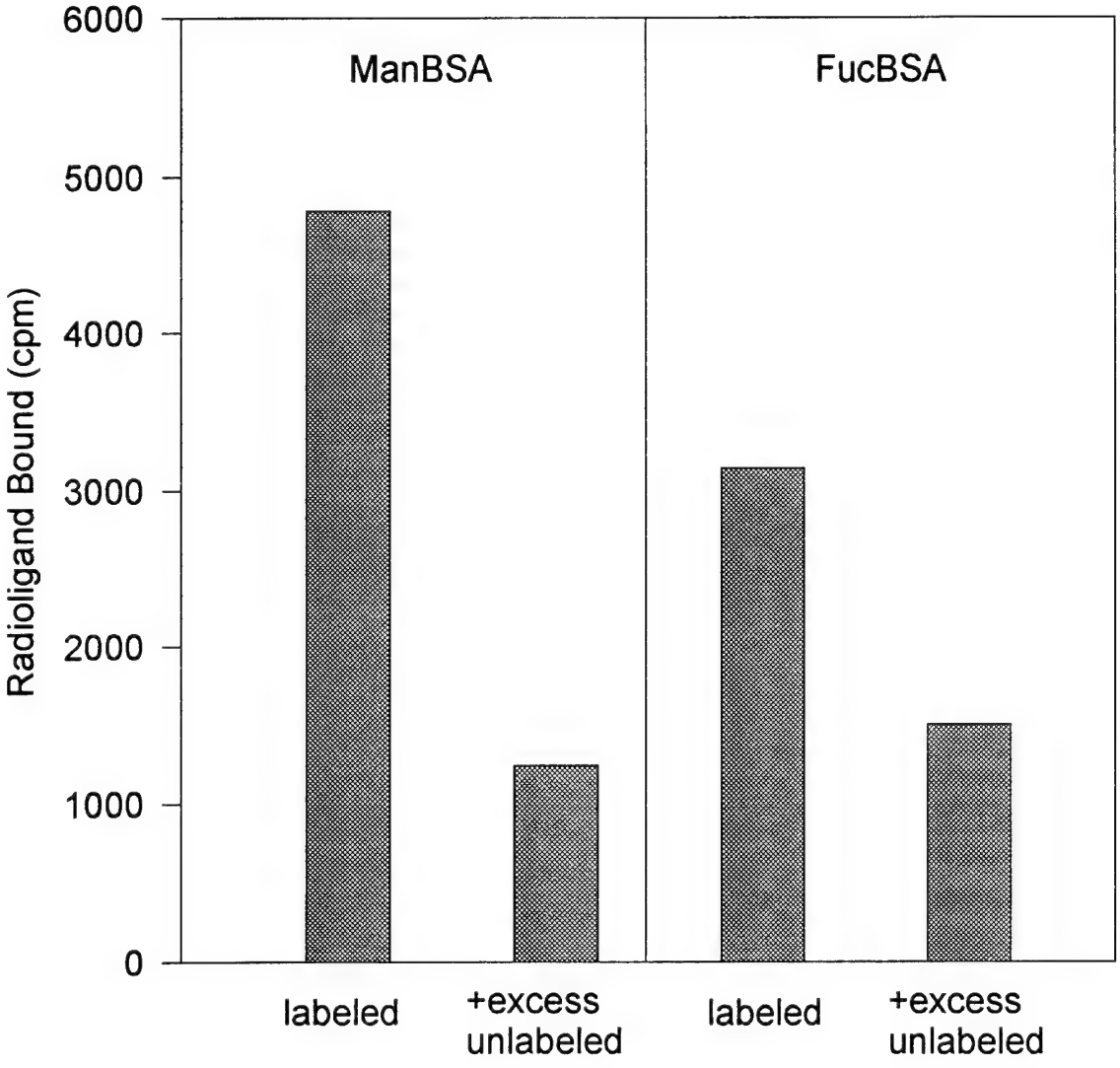
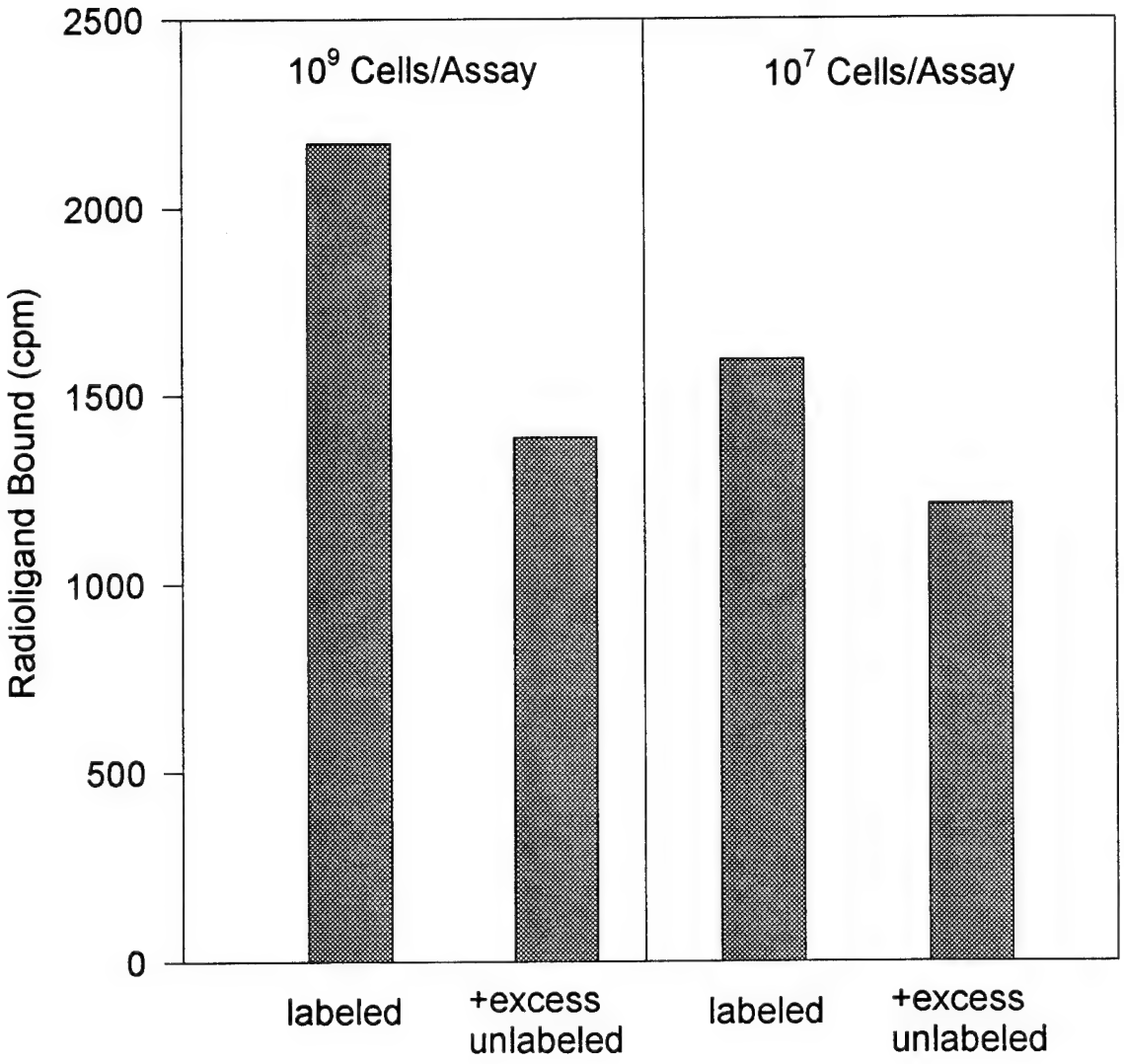


Figure 3: Radioligand Binding of ManBSA
Effect of excess unlabeled ligand



CHAPTER 2: *Entamoeba histolytica* Binding To Carbohydrates

Statement Of Problem

Amebiasis is a parasitic infection caused by *Entamoeba histolytica*, that results in 40-50 million cases of amebic colitis and liver abscess and 40,000-100,000 deaths annually worldwide (1). An initial step in parasite colonization of the large bowel is adherence to the colonic wall. Adherence involves recognition of colonic mucin and epithelial glycoconjugates by an amoebic cell surface lectin which recognizes non-reducing terminal Gal and GalNAc residues (reviewed in (2)). The goal of the current project was to characterize multivalent spaced carbohydrates as high affinity ligands for the *E. histolytica* lectin, with the goal of using multivalent carbohydrates as a basis to develop highly efficient and selective devices for pathogen capture in the field.

In prior studies funded by the ARO, a series of natural and synthetic monovalent and multivalent carbohydrate ligands was screened for inhibition of *E. histolytica* lectin-mediated human red cell hemagglutination. This screen revealed that: (i) the synthetic multivalent neoglycoprotein $GalNAc_{39}BSA$ (having an average of 39 GalNAc residues linked to lysines on bovine serum albumin) is among the most potent ligands tested, with an affinity 140,000-fold higher than monovalent GalNAc and 500,000-fold higher than monovalent Gal; and (ii) small synthetic multivalent ligands which bind with high affinity to the mammalian hepatic lectin (which has similar monosaccharide specificity) do not bind with high affinity to the *E. histolytica* lectin, revealing a distinct difference in preferred spacing of carbohydrate determinants for binding to the two lectins. The high affinity of $GalNAc_{39}BSA$ allowed facile radioligand binding studies, revealing saturable binding of ^{125}I-$GalNAc_{39}BSA$ to *E. histolytica* membranes ($K_D = 10 \pm 3$ nM, $B_{max} = 0.9 \pm 0.08$ pmol/mg membrane protein). Maximal *E. histolytica* lectin binding required either the presence of a low concentration of calcium chloride (300 μM) or a high concentration (50 mM) of sodium chloride, and had a broad pH maximum (pH 6-9). GalNAc was 7-fold more potent than Gal in blocking radioligand binding, while $GalNAc_{39}BSA$ was 160-fold more potent than $Gal_{40}BSA$. The presence of a hydrophobic aglycon (*p*-nitrophenyl β-N-acetylgalactos-aminide) enhanced affinity 8-fold compared to the free monosaccharide, and the β glycoside was a 2-fold better inhibitor than the α glycoside. When synthetic polyvalent saccharide-derivatized linear polymers were tested as inhibitors, the (GalNAcβ) and (GalNAcα3Galβ) derivatives were the most potent, with (GalNAcα) and (GalNAcα3(Fucα2)Galβ) derivatives much weaker inhibitors. The data support a model in which a unique pattern of spaced multiple GalNAc residues are the highest affinity targets for the *E. histolytica* lectin. These studies were published (3), and the reprint previously submitted to the ARO.

In extending these findings, we performed two additional studies: (i) The minimum density of GalNAc residues on polyvalent GalNAc-BSA was determined using a series of well characterized multivalent conjugates; and (ii) The role of the hydroxyl groups on GalNAc for binding to *E. histolytica* membranes was determined using deoxy derivatives as inhibitors of multivalent GalNAc radioligand binding.

Materials and Methods

Materials -- Neoglycoproteins were synthesized by the method of Lee, et al. (4). Synthetic deoxyglycosides were synthesized by Dr. R.T. Lee. Phosphate buffered saline (PBS) was a modification of Dulbecco's formulation, containing 154 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.7 mM $CaCl_2$, and 0.5 mM $MgCl_2$.

Membrane Preparation -- *E. histolytica* strain HM1:IMSS trophozoites were grown as reported previously (5). Amoebae from 3-4 250-ml flasks were collected by centrifugation (150 x *g*, 15 min, 4 °C), resuspended in 75 mM Tris, 65 mM NaCl, pH 7.2, collected as above, and resuspended in Lysis Buffer containing 10 mM sodium phosphate (pH 8), 2 mM phenylmethylsulfonylfluoride, 5 mM EDTA, 0.1 M 4-(2-aminoethyl)-benzenesulfonylfluoride, and 2 mM *p*-hydroxymercuribenzoic acid. After incubation for 5 min at 37 °C to promote lysis, the suspension was chilled on ice and sonicated (3-5 10 sec bursts, micro tip, maximum energy, Sonifier Cell Disruptor). Membranes were collected by centrifugation (50,000 x *g*, 1 h, 4 °C). The pellets were resuspended in 10 ml of Lysis Buffer and membranes collected by centrifugation (100,000 x *g*, 1 h, 4 °C). The resulting pellets were stored for up to 48 h on ice, then were resuspended in 10 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.4) containing 2 mM phenylmethylsulfonylfluoride, 2 mM EDTA and 1 mM *p*-hydroxymercuribenzoic acid and subjected to further sonication (10 10-sec bursts over a 20 min period). Membranes were collected by centrifugation as above and resuspended in 10 mM Hepes buffer pH 7.4 containing 1 mg/ml bovine serum albumin (BSA). Aliquots of the membrane preparation were stored at -20 °C until use.

Radioligand-Membrane Binding -- $GalNAc_{39}BSA$ was radioiodinated using carrier free $Na^{125}I$ and Iodobeads (Pierce Chemical Co., Rockford, IL), and the radiolabeled product was purified by gel filtration chromatography on Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ). For most experiments, the specific activity ranged from 250-500 μCi/nmol.

The standard radioligand binding reaction contained 10 mM Hepes buffer pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml BSA, and the indicated concentrations of ^{125}I-$GalNAc_{39}BSA$, *E. histolytica* membranes, and any potential saccharide inhibitor in a total volume of 100 μl. Reactions were incubated with gentle agitation for 4-5 h at 4 °C, then rapidly diluted with 10 mM Hepes buffer (pH 7.4) and rinsed onto glass fiber filters (presoaked in 10 mM Hepes buffer (pH 7.4), 1 mg/ml BSA) using a Brandel Harvester (Brandel, Gaithersburg, MD). Membrane-bound radioligand remaining on the glass fiber filters was quantitated using a γ-radiation counter.

Results

Multivalent GalNAc-BSA conjugate inhibition of $GalNAc_{39}BSA$ binding to E. histolytica membranes -- GalNAc-BSA conjugates were synthesized to have between 7 and 31 GalNAc glycosides covalently bound per BSA molecule. These were added at various concentrations to the standard assay measuring ^{125}I-$GalNAc_{39}BSA$ binding to *E. histolytica* membranes. The results, shown in *Fig. 4*, indicate that conjugates bearing between 20 and 31 GalNAc residues per BSA were indistinguishable in their high affinity for the GalNAc/Gal lectin. In contrast,

conjugates with fewer GalNAc residues had lower affinities, demonstrating the importance of multivalence for binding and the specific minimum density required.

Inhibition of $GalNAc_{39}BSA$ binding to E. histolytica membranes by deoxy GalNAc sterioisomers -- The allyl glycosides of GalNAc, 3-deoxy GalNAc and 4-deoxy GalNAc were synthesized to determine whether the 3- or 4-position hydroxyl, or both, were essential for binding to the *E. histolytica* lectin. The results are shown in *Fig. 5*. Whereas the allyl glycoside was *more* potent than the parent GalNAc, *both* deoxy derivatives were unable to inhibit radioligand binding to the *E. histolytica* lectin.

Conclusions

(i) Clustering of GalNAc residues is essential to high affinity binding to the *E. histolytica* lectin. Using the closely clustered BSA carrier, a minimum of ≈20 GalNAc glycosides per conjugate is necessary and sufficient for maximum affinity. (ii) Both the 3 and 4 position hydroxyls are required for binding of GalNAc to the lectin.

Literature Cited

1. Walsh, J.A. (1986) "Problems in recognition and diagnosis of amebiasis: Estimation of the global magnitude of morbidity and mortality," *Rev. Infect. Dis.* **8,** 228-238.

2. McCoy, J.J., Mann, B.J., and Petri, W.A.J. (1994) "Adherence and cytotoxicity of *Entamoeba histolytica* or how lectins let parasites stick around," *Infect. Immun.* **62,** 3045-3050.

3. Adler, P., Wood, S.J., Lee, Y.C., Lee, R.T., Petri, W.A.J., and Schnaar, R.L. (1995) "High affinity binding of *Entamoeba histolytica* lectin to polyvalent N-acetylgalactosaminides," *J. Biol. Chem.* **270,** 5164-5171.

4. Lee, Y.C., Stowell, C.P., and Krantz, M.J. (1976) "2-Imino-2-methoxyethyl 1-thioglycosides: New reagents for attaching sugars to proteins," *Biochemistry* **15,** 3956-3963.

5. Petri, W.A.J. and Schnaar, R.L. (1995) "Purification and characterization of the galactose- and N-acetylgalactosamine-(Gal/GalNAc) specific adherence lectin of *Entamoeba histolytica*," *Methods Enzymol.* **253,** 98-104.

Figure 4: Inhibition of ^{125}I-GalNAc$_{39}$BSA Binding to *E. histolyitca* membranes by multivalent GalNAc-BSA conjugates

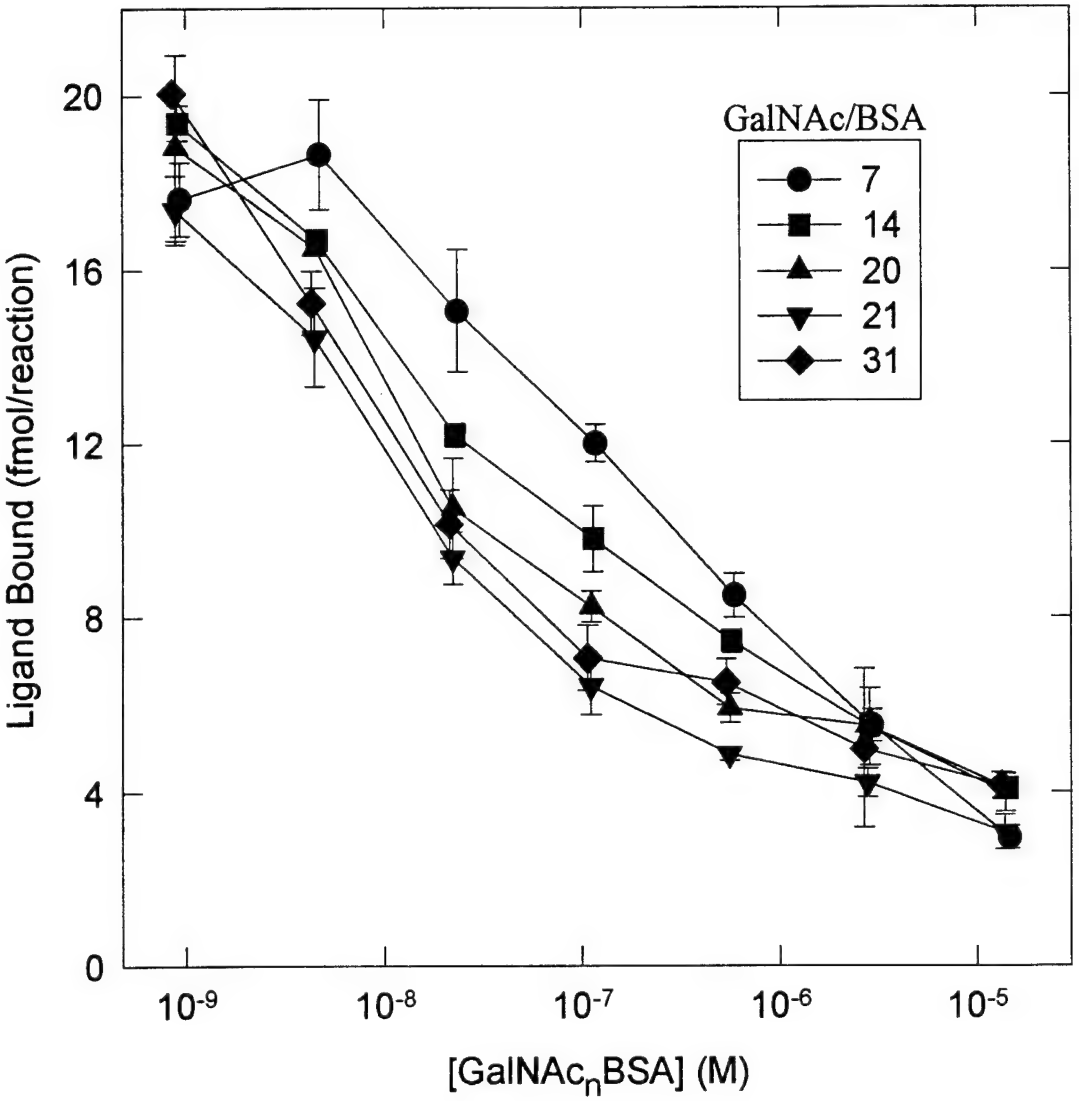
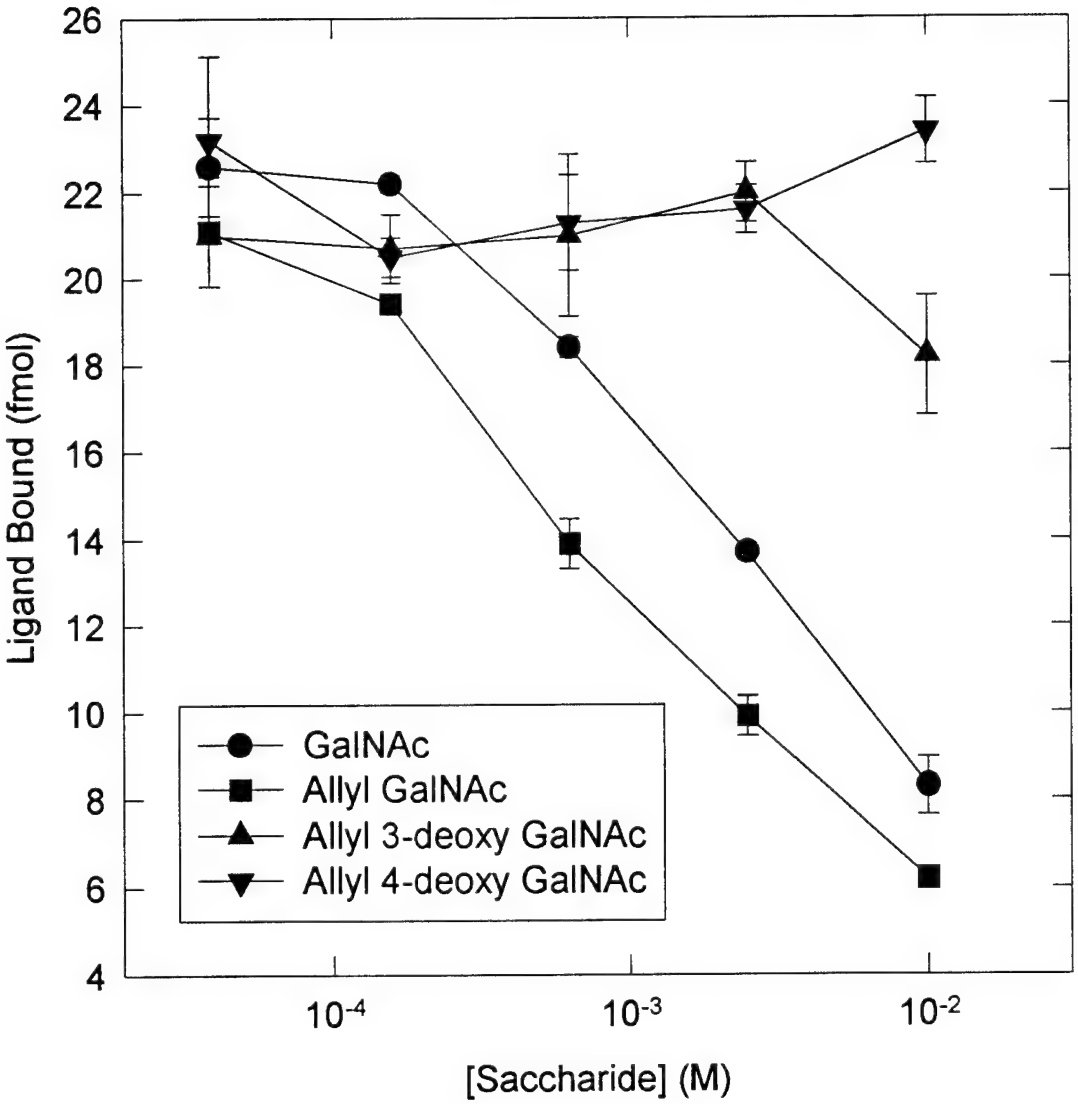


Figure 5: Inhibition of ^{125}I-GalNAc$_{39}$BSA Binding to *E. histolyitca* membranes by GalNAc derivatives



CHAPTER 3: *Entamoeba histolytica* Hexosaminidase

Statement Of Problem

During analysis of the specificity of the *E. histolytica* lectin, a variety of glycosides were tested, including *p*-nitrophenyl (pnp) glycosides. A surprising finding which arose was that pnp-GlcNAc, while it did not inhibit binding to the lectin, was readily cleaved by the *E. histolytica* membranes. This indicated the presence of a hexosaminidase in the membranes. Since such a hexosaminidase may be involved in the production of binding sites (underlying GalNAc residues) on target cell surfaces, the characterization of the enzyme was undertaken.

Methods

Enzyme activity was determined in microwells in which enzyme source and pnp-glycosides were incubated in phosphate-buffered saline at 37°C. At desired times, the production of yellow color (glycosidase activity) was determined using a microplate spectrophotometer with filters set to read A_{405}.

E. histolytica membranes were isolated as described in Chapter 2. Membranes were extracted in 10 mM sodium phosphate buffer (pH 7) containing 30 mM octylglucoside and protease inhibitors (EDTA, leupeptin, pepstatin, PMSF and aprotinin). Residual membranes were removed by centrifugation (75,000 x *g*, 30 min) and the supernatant stored at O°C.

Column chromatography was performed on a TSK DEAE-5PW HPLC ion exchange column or on a custom-packed hydroxyapetite column as indicated. The ion exchange column was loaded in 10 mM sodium phosphate buffer (pH 7), 30 mM octylglucoside, and was eluted in the same buffer containing a gradient of NaCl from zero to 1 M. The hydroxyapetite column was loaded in the same buffer, then eluted with a gradient from zero to 0.5 M potassium phoshate pH 7. SDS-PAGE was performed according to the methods of Laemmli (1).

Results

A specific hexosaminidase was detected in *E. histolytica* membranes. It was released only by detergent treatment (extensive sonication did not remove it, nor did washes with salt-containing solutions). The solubilized enzyme was purified 128-fold with 30% recovery (*Table V*).

Kinetic analysis revealed a well-behaved enzyme-substrate interaction resulting in a rectangular hyperbola when enzyme velocity was plotted against the substrate concentration (*Fig. 6, top*). Linearization of the data using an Eadie-Hoffstee plot (*Fig. 6, bottom*) revealed a K_M of ≈200 μM.

Specificity studies revealed a highly specific β-glucosaminidase (*Fig. 7, top*). Activity against the α-anomer was negligable, as were activities against Glc, Gal, and Man glycosides. Activity against pnpβGalNAc was <10% of that against pnpβGlcNAc (*Fig. 7, bottom*).

Activity was not irreversibly denatured when run on SDS-PAGE (data not shown). Electrophoresis with molecular weight standards, followed by incubation of the resulting gel in a solution of pnpβGlcNAc revealed production of cleavage product (yellow color) migrating at 97,000 daltons.

Conclusions

A highly specific, stable, and large molecular weight hexosaminidase is associated with *E. histolytica* membranes. Its potential functional role in carbohydrate recognition by the microbe has yet to be determined.

Literature Citation

1. Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* **227**, 680-685.

TABLE V

Fraction	protein (mg)	Activity ΔA405/hr	recovery	Specific Activity	purification
E. Histolytica membranes	68.9	10,928	100%	159	1.0
Detergent extract	22.5	8,256	76%	366	2.3
DEAE 5PW - 1	1.81	5880	54%	3,251	20.5
DEAE 5PW - 2	0.56	4,371	40%	7,747	48.9
Hydroxyapetite	0.160	3,245	30%	20,296	128.0

Figure 6: Kinetics of *E. histolytica* hexosaminidase

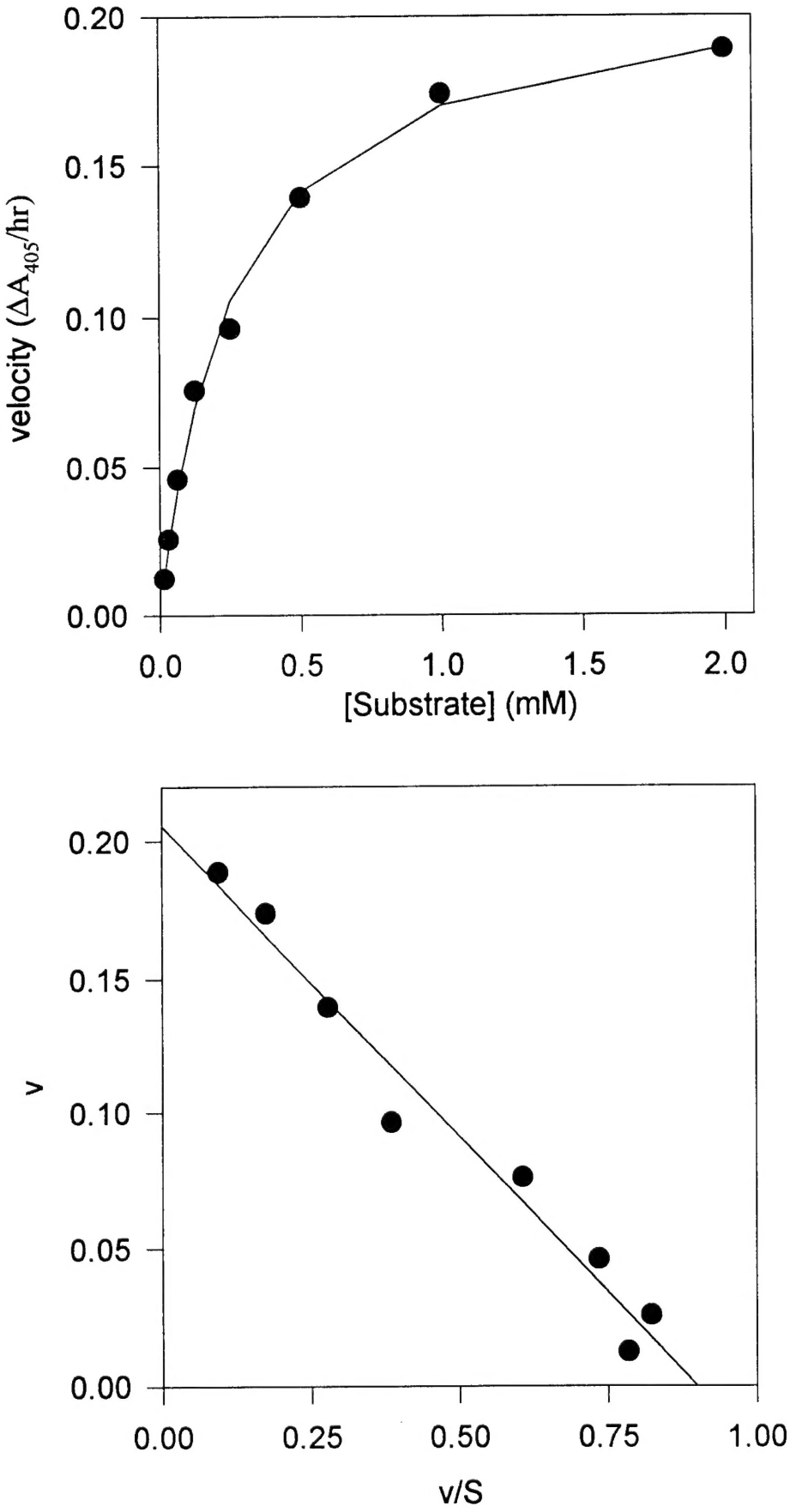
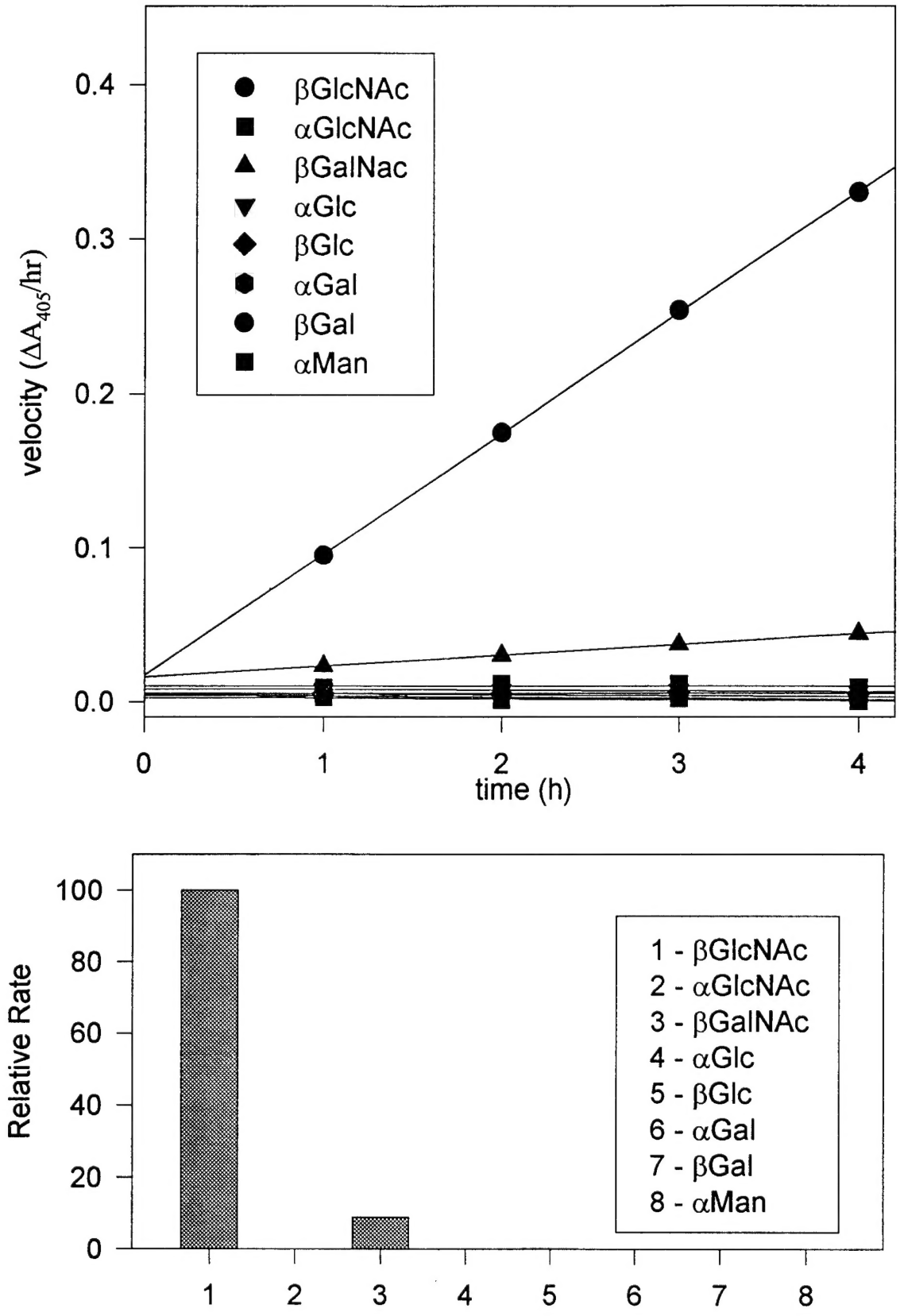


Figure 7: Specificity of *E. histolytica* hexosaminidase



Publication

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